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Matters Arising

P. falciparum CG2, Linked to Chloroquine Resistance, Does Not Resemble Na^+/H^+ Exchangers

Understanding the molecular basis for chloroquine resistance in *Plasmodium falciparum* will provide important support for the development of new therapies and prophylactic measures against malaria. Complementary genetic and biochemical investigations should discriminate among current theories and pinpoint the functional determinants of resistance. With this in mind, Sanchez et al. (1998) have proposed that the *P. falciparum cg2* gene, linked by Su et al. (1997) to chloroquine resistance, may encode a sodium/hydrogen exchanger (NHE) responsible for drug transport. Here, we present evidence against this proposal. Detailed reanalysis of the CG2 sequence fails to support the claims for significant similarity to functional features of well-characterized eukaryotic NHE transport domains.

First, although the CG2 sequence has some clusters of hydrophobic amino acids, these are not typical of known types of integral membrane proteins, including those from *P. falciparum*. Of the seven potential transmembrane segments annotated on the CG2 sequence in Figure 3 of Su et al. (1997), six gave only marginal predictions in the three algorithms used. Figure 1 shows results from two additional well-validated algorithms, TMHMM (Sonnhammer et al., 1998) and MEMSAT (Jones et al., 1994), which evaluate potential transmembrane helices together with characteristic flanking loop sequences. When applied to known mammalian NHE sequences, both methods successfully predict 10–12 transmembrane regions and much of their known connectivity topology, yet these methods give negative results for the CG2 sequence (Figure 1). Hydrophobicity profiles of NHE proteins show large peaks, whereas CG2 shows, at most, only marginal spikes of local hydrophobicity (Figure 1). It is an open question which, if any, of these short, widely separated hydrophobic clusters in CG2 have the potential to span a membrane. The presence of more than seven transmembrane segments, as occurs in known exchanger proteins, is highly improbable.

Second, the sodium/hydrogen ion transport domain homology proposed by Sanchez et al. (1998), and the results of database searches, have been reanalyzed by established statistical methods (Altschul et al., 1994; Altschul and Gish, 1996). No pairwise alignment scores between the proposed CG2 domain and known NHE transport domains approach even borderline statistical significance. All of these scores have a 0.8 probability or greater of occurring in random pairwise alignments of protein sequences with the same sizes and compositions. Moreover, the proposed CG2 domain shows considerably stronger (but still nonsignificant) scores in alignments with many proteins in current databases that are not transporters.

Third, Sanchez et al. (1998) claim that the motif VFFLFSI in CG2 resembles the characteristic amiloride binding signature VFFLFLL in NHE sequences. They give a probability of 8×10^{-4} for the chance occurrence of VFFLF in a random sequence of CG2's size and composition. However, the actual chance of finding a similar or stronger match to VFFLFLL in *P. falciparum* proteins is much greater than this because of the strong, nonrandom, compositional bias and amino acid clustering characteristic of this parasite. The residues encoded by AU-rich codon sets (such as F, L, I, N, K, and Y) in *P. falciparum* are unusually abundant, and these amino acids also have a strong tendency to occur clustered together in low complexity regions. An analysis of 729 nonidentical *P. falciparum* amino acid translations available from public databases showed 126 clusters ($\geq$10 contiguous amino acids) containing at least 70% F/L. To avoid bias in this analysis, candidate N-terminal hydrophobic signal sequences were excluded from the count. Therefore, the VFFLF match in CG2 is not surprising, and there is no statistical reason for attributing special functional significance to this motif.

Additionally, our immunoelectronmicroscopy studies do not show the plasma membrane localization that would be expected if CG2 were an integral membrane NHE. Figure 5 of Su et al. (1997) showed that CG2 in

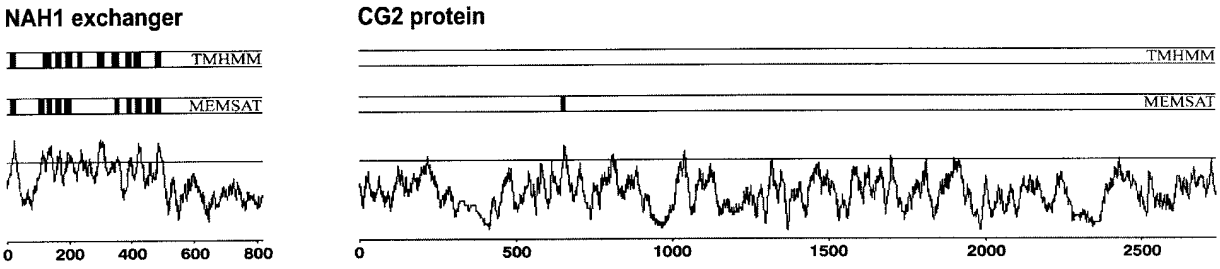

Figure 1. Hydrophobicity and Transmembrane Analyses Demonstrate Substantial Differences between the CG2 Protein and Typical NHE Exchangers

Average hydrophobicity was plotted over a 17-residue window (Kyte-Doolittle parameters) for the human amiloride-sensitive sodium/hydrogen exchanger NAH1 (GenBank protein sequence ID *178753*, Swiss-Prot P19634; other NHE sequences not shown give similar results) and the CG2 protein sequence of the chloroquine-resistant Dd2 clone. The horizontal line intersecting each curve represents an accepted diagnostic threshold typically exceeded by candidates for transmembrane helices. The solid blocks above the curves represent the transmembrane helices predicted by TMHMM and MEMSAT. Scale bars indicate amino acid sequence numbers.

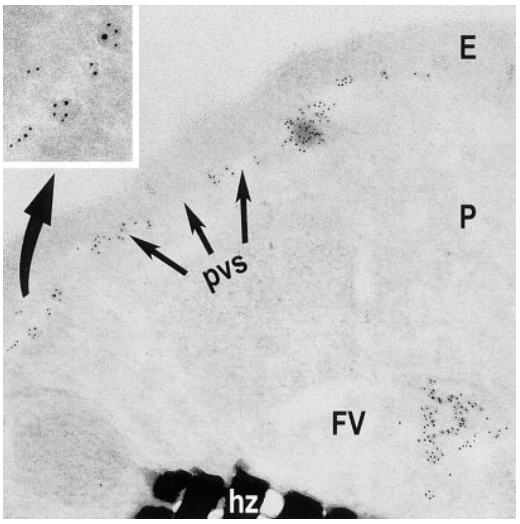

Figure 2. Immunoelectron Micrograph Showing Location of CG2 Protein within a *P. falciparum*-Infected Human Erythrocyte

Inset shows vesicle-like structures localized at the parasitophorous vacuolar space (pvs) delineated by the parasite plasma membrane and the surrounding parasitophorous vacuolar membrane. Similar structures are evident in the vicinity of the large parasite food vacuole (FV) containing hemozoin (hz). E, erythrocyte; P, parasite (schizont stage). Immunoelectronmicroscopy was as described (Su et al., 1997), except that the B4D12 monoclonal antibody was purified on a protein A/G column and detected with goat anti-mouse IgG conjugated to 5 nm gold particles (Amersham International Plc.).

trophozoite and schizont stages is detected at the parasitophorous vacuolar space surrounding the intraerythrocytic parasite and within parasite food vacuoles containing hemozoin pigment (β-hematin polymers). At the parasitophorous vacuolar space, CG2 is localized to vesicle-like structures, not the plasma membrane, as shown clearly by high detail immunoelectronmicroscopy (Figure 2). The data also show that CG2 is distributed within parasites in similar structures, which have an electron density comparable to or greater than that of the hemoglobin-filled cytoplasm of parasitized erythrocytes.

Leading theories of chloroquine resistance account in different ways for the ability of resistant parasites to overcome the toxicity resulting from chloroquine binding to the hematin liberated during digestion of host cell hemoglobin in acid vesicles (Ridley, 1998). Theories of drug transport developed over the past decade have variously postulated enhanced efflux or reduced influx of chloroquine by modified transporters localized in cytoplasmic or vesicular membranes. Yet impressive validity remains in older theories (Fitch, 1969, 1970) that explain resistance in terms of a change in chloroquine–hematin binding interactions.

Proponents of these different theories of modified high affinity binding (Fitch, 1969, 1970; Bray et al., 1998) or modified transporter properties (Krogstad et al., 1987; Sanchez et al., 1997) have made detailed measurements of chloroquine uptake and release in parasitized erythrocytes following treatment of drug-resistant and drug-sensitive lines with different concentrations of chloroquine. The independent yet remarkably similar data sets show simple saturation effects (for example, linear Scatchard or Hill plots) in the nanomolar range of extracellular chloroquine, but such data alone cannot distinguish between the formal kinetic schemata of active transport or intracellular sequestration. An altered association constant of chloroquine–hematin binding or reduction of chloroquine availability to hematin via a non–membrane-dependent mechanism, as supported by the experiments of Bray et al. (1998), would be consistent with a resistance factor that acts in direct association with hematin inside vesicles where CG2 is found (Su et al., 1997).

CG2 may thus affect chloroquine access, processes related to hemoglobin digestion, heme sequestration, or the toxicity of hematin–chloroquine complexes. Investigations including gene modifications and biochemical assays will be required to understand the fascinating interactions of this unique protein and its role in chloroquine resistance.

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